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Development of Serotonin-like and SALMFamide-like Immunoreactivity in the Nervous System of the Sea Urchin *Psammechinus miliaris*

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Abstract. The present immunocytochemical study utilizes serotonin and SALMFamide antisera, together with confocal laser scanning microscopy, to provide new information about the development of the nervous system in the sea urchin *Psammechinus miliaris* (Echinodermata: Echinoidea). Special attention is paid to the extent of the nervous system in later larval stages (6-armed pluteus to metamorphic competency), a characteristic that has not been well described in this and other species of sea urchin. An extensive apical ganglion appears by the 6-armed pluteus stage, forming a complex of 10-20 cells and fibers, including discrete populations of both serotonin-like and SALMFamide-like immunoreactive cells. At metamorphosis this complex is large, comprising at least 40 cells in distinct arrays. Serotonin-like immunoreactivity is also particularly apparent in the lower lip ganglion of 6- to 8-armed plutei; this ganglion consists of 15-18 cells that are distributed around the mouth. The ciliary nerves that lie beneath the ciliary bands in the larval arms, the esophagus, and a hitherto undescribed network associated with the pylorus all show SALMFamide-like immunoreactivity. The network of cells and fibers in the pyloric area develops later in larval life. It first appears as one cell body and fiber, then increases in size and complexity through the 8-armed pluteus stage to form a complex of cells that encircles the pylorus. SALMFamide-like, but not serotonin-like, immunoreactivity is seen in the vestibule wall, tube feet, and developing radial nerve fibers of the sea urchin adult rudiment as the larva gains metamorphic competency.

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Introduction

The presence of a nervous system in echinoid larvae was first suggested by observations of a putative thickening in the apical area (MacBride, 1914) and the presence of neuron-like cells in the adoral ciliary band (Mortensen, 1921). Further evidence for such a system was circumstantial, derived from observations of feeding, locomotion, settlement, and orientation behavior. These behaviors, although relatively simple, suggested the coordinating influence of a nervous system, an idea finally confirmed by studies on ciliary activity (Mackie *et al.*, 1969). More recent histological and histochemical studies have provided greater details of the arrangement and fine structure of neurons in the ciliated bands and esophageal musculature of echinoderms (Burke, 1978; Nakajima, 1986, 1988; Lacalli *et al.*, 1990).

A rich quota of neurochemicals is associated with the larval nervous system, including acetylcholine, serotonin, γ-aminobutyric acid (GABA), noradrenaline, and dopamine (Bisgrove and Burke, 1987; Nakajima, 1988). Such a variety of agents hints at the organizational complexity of this system. Histochemical and immunocytochemical methods have been especially productive in revealing previously unknown neural structures in larvae (Nakajima, 1986; Kroll and Voronezhskaya, 1996). The discovery of the native echinoderm neuropeptides, S1 (GFNSALMFamide – amino acid notation) and S2 (SGPYSFNSGLTFamide), and the subsequent availability of highly specific antibody probes have provided a further means to investigate the larval and adult echinoderm nervous system.

S1 was originally isolated from starfish and shown to have a ubiquitous distribution in the nervous system of *Asterias rubens* (Elphick *et al.*, 1991a; Newman *et al.*, 1995a, b). Similar peptides that seem to be members of the same family were also identified in holothurians (Diaz-

Miranda *et al.*, 1992), and S1-like molecules are also present in crinoids and ophiuroids (Ghyoot *et al.*, 1994; Candia Carnevali *et al.*, 1998). This family of peptides has also been localized in the larval nervous systems of starfish (Moss *et al.*, 1994; Byrne *et al.*, 1999) and sand dollars (Thorndyke *et al.*, 1992), revealing subsets of neurons not previously identified with other probes.

Novel neurochemical markers (such as peptide antisera) can help us complete our picture of the larval and adult nervous systems and, perhaps, add supporting data to other studies of the physiological function of the molecules. That is, information about the location of neurochemicals within a cell, within a cell type, or within a tissue type can sometimes help to indicate whether these molecules may be involved in motor or sensory activities or may be neurotransmitters or neuromodulators. In echinoderms, neurochemical markers, when expressed at various developmental stages, can help us visualize the origin, growth, and fate of particular neural structures.

The aim of the present study was to map the development of the larval echinoid nervous system using immunocytochemical techniques, with established commercial (serotonin) and novel (SALMFamide) antisera serving as probes. We used *Psammechinus miliaris*, a small regular sea urchin of the class Echinoidea, which is common along the northeast Atlantic and North Sea coasts of the United Kingdom and Scandinavia. The development of *P. miliaris* has never been fully described in the literature, although we and other researchers (*e.g.*, Kelly *et al.*, 2000) have found it to be similar to that of other species of indirect developing sea urchins, passing through embryo, prism, and 4-, 6-, and 8-armed plutei stages to metamorphic competency (MacBride, 1913, 1914, 1918). In the current work, emphasis has been placed on the later larval stages, which are less well described by immunocytochemical studies. Confocal microscopy has been used to facilitate the accurate localization of larval neurons, to identify previously unknown components of the nervous system, and to improve the resolution of the cells and fibers.

Materials and Methods

Animal maintenance and larval culture

Specimens of *Psammechinus miliaris* were obtained from the University Marine Biological Laboratory at Millport, Isle of Cumbrae, and from Dunstaffnage Marine Laboratory, Oban, on the west coast of Scotland. They were maintained in circulating seawater systems at Royal Holloway or in Oban and fed on *Ulva lactuca*, *Mytilus edulis*, and an artificial food consisting of soya meal, fish oil, and spinach. Males and females could generally be distinguished by slight differences in the appearance of their gonopores. Animals were spawned by the injection of 0.5 *M* KCl in filtered seawater (FSW) into the area of the gonads, with the needle inserted through the peristomial membrane (Iwate and Fukase, 1964). Animals were placed in glass beakers for about 30 min, or until they spawned. Gametes were collected and eggs fertilized by standard methods (Strathmann, 1987). After hatching, larvae were maintained in autoclaved seawater in glass jars and gently stirred to prevent them from gathering at the bottom of the vessel. Once the gut had developed, the larvae were fed *Dunaliella tertiolecta* so that algae could always be seen in the gut. The larval cultures were kept clean by replacing one-third of the water three times a week. Cultured algae were separated from the growth medium and resuspended in FSW before being fed to the larvae. To prevent overcrowding, the concentration of larvae in each jar was kept at about 1 per 5 milliliters of FSW. Animals were thus maintained throughout larval development, until competency. Competent larvae with a large, well-developed rudiment were induced to settle by the introduction of algal-fouled glass plates from the aquarium. Similarly, larvae would also often settle and metamorphose on the bottom of the culture vessels, if these were allowed to become fouled with algal debris.

Fixation

Samples of larvae were removed from culture and fixed at regular intervals, ensuring that each stage (4-armed, 6-armed, and 8-armed plutei) was adequately represented. At least 100 larvae per sample were fixed at earlier stages. This reduced to 20 per sample for 8-armed animals with developed rudiments. Larvae were anesthetized in 4% $MgCl_2$ in FSW or killed by the addition of a few drops of 4% paraformaldehyde (PFA) in FSW. Excess seawater was then pipetted off, and the samples were fixed in 4% PFA in FSW for 2 h. For storage, animals were rinsed in FSW and stored in FSW containing 0.01% NaN_3 at 4°C.

Immunocytochemistry

Whole-mount incubations were carried out in 2-ml glass tubes at room temperature. Larvae were rinsed in phosphate buffered saline (PBS) and permeabilized by a 30-min incubation in 0.5% Triton-X 100 in PBS, followed by 30 min in 5% normal goat serum in PBS. To improve antiserum penetration, and thus the staining of the rudiment in older larvae, a further permeabilization step was carried out. After fixation, larvae were rapidly dehydrated to 70% ethanol, rehydrated, and incubated in 0.5% Triton-X 100 in PBS for 1-2 h. Primary antibodies were diluted in PBS, and the larvae were incubated in this solution for 16-18 h. The serotonin antiserum (SeraLabs, UK) was raised in rabbit against synthetic serotonin and used at 1:200 in PBS. All results with this antibody are referred to as serotonin-like immunoreactivity (5HT-Li IR). The peptide antisera were raised in rabbits and produced in this laboratory; they are all polyclonals. Anti-S1 (BL1V/anti-KYSALMFa) or anti-S1

(BLIIIa/anti-KYSALMFa) (Elphick *et al.*, 1991a, b) were used at 1:200 in PBS, which gave clear staining with low background. These antisera are two consecutive bleeds from one animal and will be referred to as S1-like immunoreactivity (S1-Li IR). Some 6-armed plutei were also stained for S2 with a new polyclonal raised in this laboratory (N1/anti-KYSGLTFa) (Potton, 1997), which produced preparations of high specificity and low background levels. Results with this antiserum are referred to as S2-like immunoreactivity (S2-Li IR).

After overnight incubation in primary antisera, larvae were washed in PBS, three times each for 5 min. Visualization was by a 1-h incubation in biotinylated anti-rabbit IgG (1:200 in PBS) (Vector Labs, Peterborough, UK), rinsing in PBS, and then a final incubation for 1 h in avidin-conjugated FITC or Texas red (1:100 in PBS) (Vector Labs). Larvae were rinsed in PBS and mounted in Vectashield antifade mountant (Vector Labs) and viewed on a Zeiss Axioplan with fluorescent attachments, or on a Leica TCS-4D confocal laser scanning microscope (CLSM).

Controls were produced by preabsorption of twice the working concentration of primary antiserum with an equal volume of 2×10 μM KYSALMFa, KYSGLTFa, or 5HT (Polak and Van Noorden, 1986). This reduced staining to background levels in all cases, although it was not completely removed. Preadsorption controls revealed weak cross-reactivity between the peptide antisera where preadsorption of anti-S1 with S2 did not affect staining as much as did preadsorption of anti-S2 with S1. Further controls for secondary antibodies were carried out by replacing the primary with PBS, or by using direct visualization with FITC- or Texas red-conjugated secondary antibodies, which controlled for reactions with endogenous biotins.

Results

For the purposes of this study, the development of *Psammechinus miliaris* was divided into five stages: prism (2 to 3 days post-fertilization), 4-armed, 6-armed, and 8-armed plutei (from 3 to 20 days), and competent plutei (from 20 to 30 days). The adult rudiment began to form at the 6-armed stage, and was well developed in the late 8-armed pluteus. In cultures of *P. miliaris* the total development period, from egg to new juvenile, was between 20 and 30 days. Figure 1 shows the key features of the pluteus nervous system and summary diagrams of the results. Figure 1A depicts the extent of the larval nervous system as it is known from previous immunocytochemical studies. Figure 1B shows the more extensive system revealed by the present immunocytochemical study.

Observations of living larvae revealed distinctive cell types associated with the sea urchin larval nervous system. These observations, together with previous descriptions of neuronal cell types in these areas (Burke, 1978; Nakajima, 1986; Lacalli *et al.*, 1990), assisted us in identifying those cells in *P. miliaris* that were stained by immunocytochemical methods. In late prism larvae (2-3 days post-fertilization), the ciliated band could be clearly observed. The cells of the blastocoelar network were also visible (Fig. 2A). These cells were previously thought to be part of the nervous system, but subsequent work has shown that they are not neural but have some other function, possibly associated with muscle activity (Burke, 1978). Neuron-like cells within the ciliary bands were visible; their characteristic shape, with a thick apical process, suggested they might be sensory cells (Fig. 2B, C). Fine basal processes contribute to a tract of fibers beneath the ciliary band, forming part of the ciliary nerve (Fig. 2C), which then putatively connects to other larval structures. The ciliary band and its associated neurons and fibers elongates as the larval arms develop and form the main neural pathways of the larva. Along with the apical ganglion, lower lip ganglion, and neural components of the gut, these structures constitute the main neuronal elements of the sea urchin larval nervous system (Fig. 1).

The immunocytochemical studies focused on later pluteal stages, which have been less thoroughly described in previous investigations (Bisgrove and Burke, 1987; Thorndyke *et al.*, 1992). These studies enabled a detailed examination of the fully developed apical ganglion and adult rudiment.

Development of immunoreactivity in plutei to the 8-armed stage

The earliest cellular labeling observed in young plutei was a single cell within the thickened lower lip of the stomatodeum, which was strongly immunoreactive to the serotonin antibody (Fig. 3A). This cell was seen in the most precocious larvae at about 72 h post-fertilization. The cell has a basal unlabeled nucleus and faintly immunoreactive basal processes that extended beneath the lip. There was also a suggestion of labeled cells in the ciliated band of the anterolateral arm buds. There was no comparable labeling with the other antisera in larvae of the same age or in control specimens that received only primary antibody or an inappropriate secondary antibody. At the fully developed 4-armed stage (4 or 5 days post-fertilization) the lower lip contains several cells with 5HT-Li IR in a symmetrical arrangement (Fig. 3B). A similar pattern of labeled lower lip cells is also apparent in 4-armed specimens stained with anti-S1, although these cells are comparatively large (15 μm), and less numerous. At this stage, no S1-Li IR cells were seen in the apical thickening. The larval stomach contains algae and is autofluorescent.

By the 6- to 8-armed pluteus stage, the number of labeled cells in the lower lip complex is increased, with up to 18 neurons showing 5HT- and S1-Li IR. When viewed from the side, these cells have a characteristic shape, with broad bases against the esophageal epithelium and narrower api-

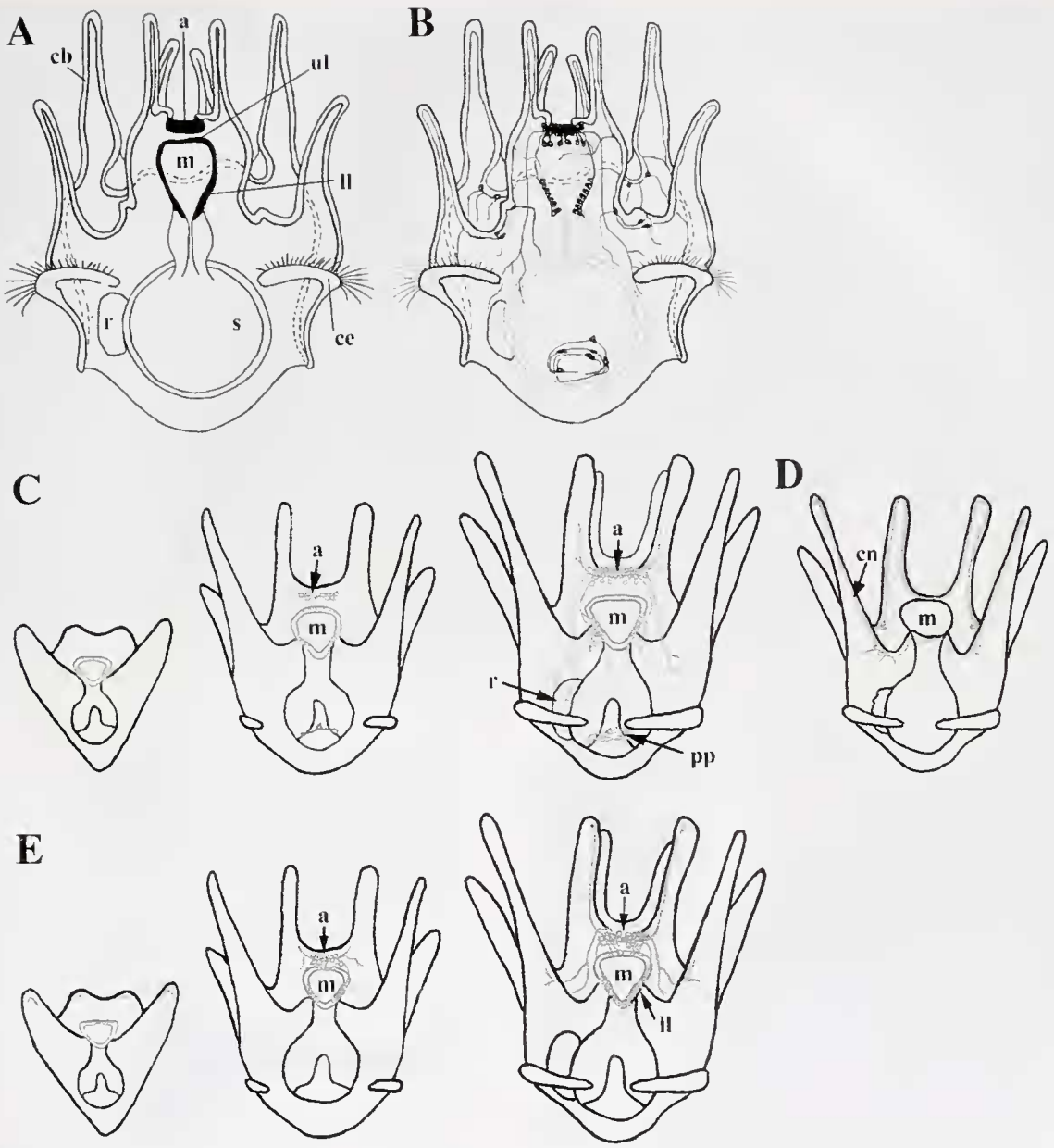

Figure 1. Diagrams of pluteus larvae. (A) Previously demonstrated sites of innervation (solid fill) in a sea urchin larva (Nakajima, 1986; Bisgrove and Burke, 1987; Thorndyke *et al.*, 1992). (B) The extent of the nervous system, as revealed in this study. (C) The main areas of S1-Li IR (gray) in the early 4-armed, early 6-armed, and 8-armed pluteus. (D) The main areas of S2-Li IR (gray) in the 6-armed pluteus. (E) The main areas of 5HT-Li IR (gray) in the early 4-armed, early 6-armed, and 8-armed pluteus, clearly showing its predominantly ventral distribution. a-apical ganglion, cb-ciliary band, ce-ciliary epaulette, cn-ciliary nerve, ll-lower lip, m-mouth, pp-pyloric plexus, r-rudiment, s-stomach, ul-upper lip. Not to scale.

ces at the ectoderm of the inner edge of the oral hood. From the 6-armed stage onwards the apical complex begins to increase dramatically in both size and complexity, whereas the lower lip complex expands no further. Indeed, the 5HT-Li IR appears to weaken at later stages. The row of small cells first seen in the upper lip at the 4-armed stage alters little during subsequent development.

In later plutei, 5HT-Li IR cells in the upper lip could be seen contributing processes to the expanding apical ganglion (Fig. 4A-D). This association is close enough at this stage for the apical and upper lip complexes to be considered part of the same ganglion. The apical complex becomes the predominant site of both 5HT and S1-Li IR neurons, as the 4- and 6-armed plutei progresses to a full 8-armed stage,

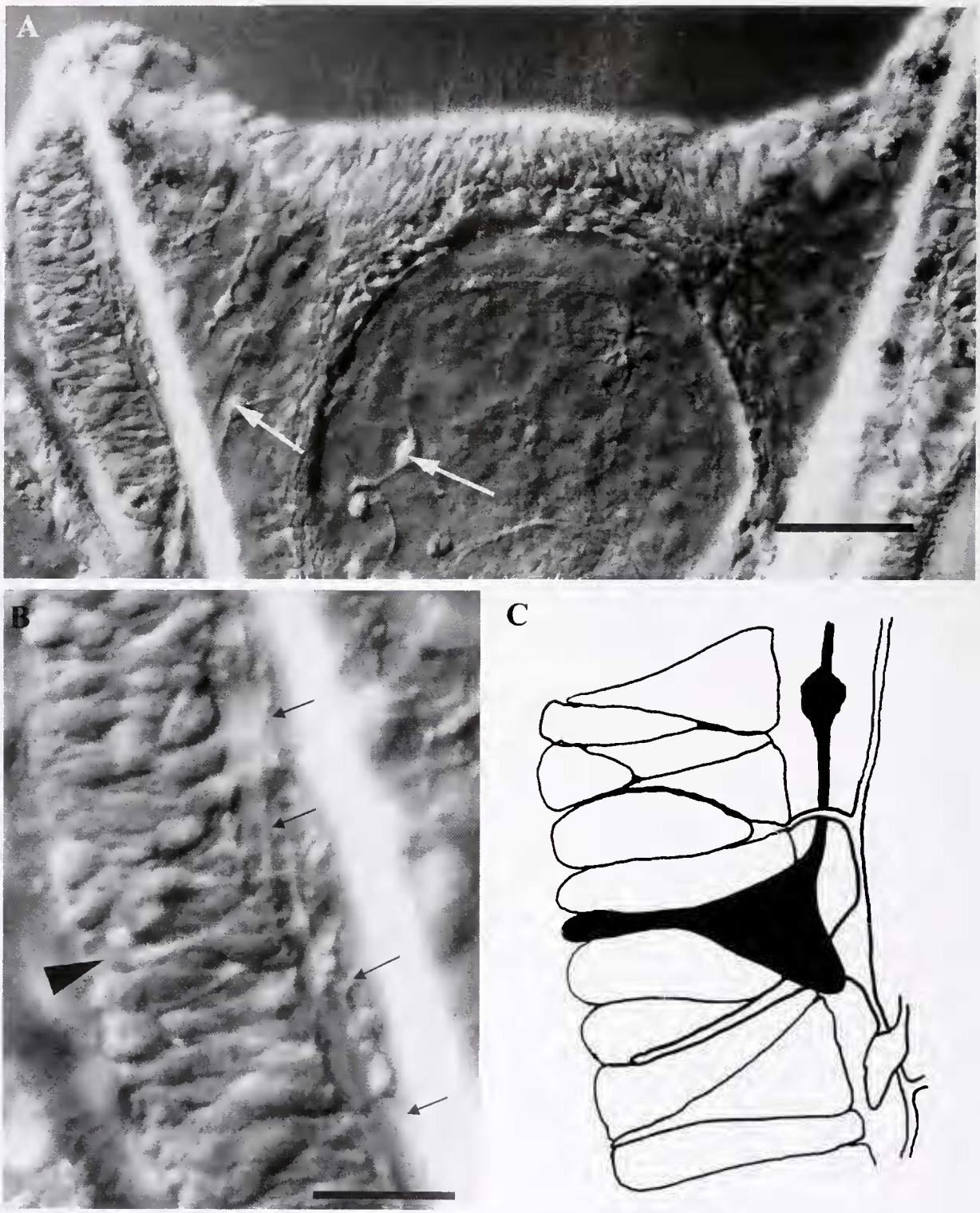

Figure 2. Observations of live larvae. (A) Multipolar cells of the blastocoelar network in the oral hood of an early 4-armed pluteus (arrows). (B) Higher magnification of (A) showing a flask-shaped bipolar neuron with a tapering apical process (arrowhead) and basal varicose axon contributing to the ciliary nerve (arrows). (C) Diagram of (B) with the neuron shown in black (not to scale). Bars: A = 25 μm, B = 10 μm.

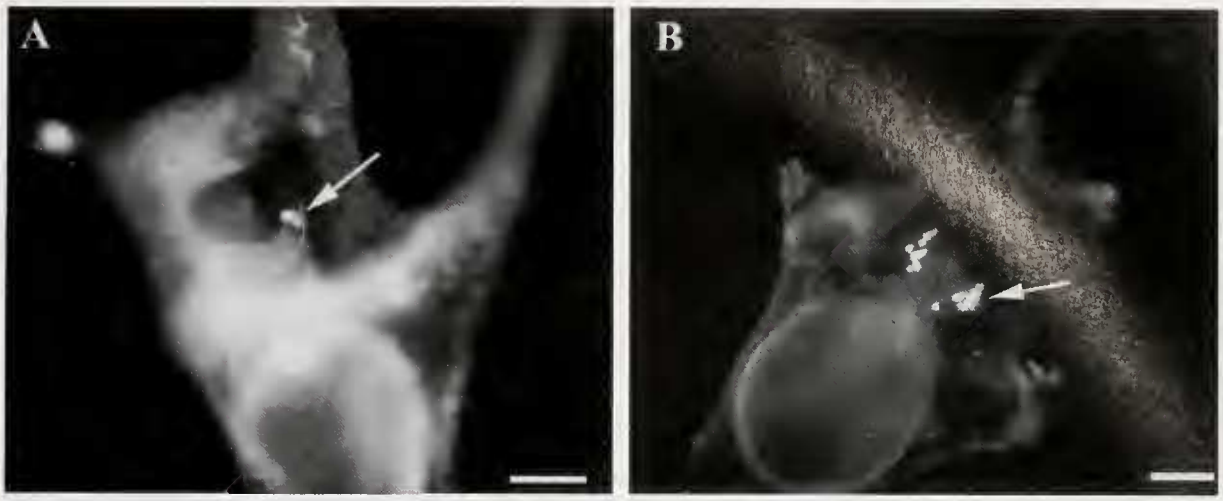

Figure 3. Immunoreactivity in the lower lip ganglion. (A) Late prism larva with a single serotonergic cell in the lower lip (arrow). (B) Lower lip ganglion with 10-12 cells showing 5HT-Li IR (arrow). Bars = 50 μm.

with cells being added rapidly through this period (Fig. 4A, B, D).

In fully developed 6- and 8-armed plutei, the differences in distribution between peptide and serotonin immunoreactivity become more apparent. The ciliary nerves show S2-Li IR, rather than S1-Li or 5HT-Li IR (Fig. 5A). These axons possess varicosities and some immunoreactive cell bodies associated with the descending loops of the ciliary bands (Fig. 5B, C). The neuronal cell bodies are generally multipolar, rounded or elliptical, and with large nuclei (Fig. 5B). Unlike the cells of the apical ganglion, these cells have no obvious sensory processes or contacts with ectodermal surfaces. No 5HT-Li IR was found in these areas.

Development of immunoreactivity in late plutei (8-armed stage to metamorphosis)

As the plutei grow and the rudiment forms, changes in the nervous system or in the distribution of neurotransmitters become more difficult to observe with traditional epifluorescence microscopy. However, scanning confocal laser microscopy allowed us to examine these changes in detail, especially in relation to the apical ganglion complex, which became very large and convoluted in older larvae.

The apical ganglion continues to increase in size and complexity until the mature 8-armed stage. In these older larvae, it is as much as 80 μm in length (left to right side of the larva) and 60 μm deep, extending from the thickened apical ciliated band down to the upper lip. To resolve this cluster of perikarya and dense neuropile, rotations were calculated for three-dimensional confocal images (Fig. 6). Some double-labeling studies were attempted, but due to the large size of the specimens and the large number of cells in the ganglion, the results obtained were not clear and have therefore not been presented here. However, confocal images of separately stained preparations were informative and suggested that the distributions of S1 and 5HT-Li IR within the apical ganglion differed significantly. Some cells may have expressed both substances, and there may have been other cell types present that expressed neither, but overall the rotational images provided the most comprehensive view of this structure to date.

In mature larvae, about 24 cell bodies in the apical ganglion show 5HT-Li IR. These are arranged symmetrically on either side of a dense central plexus, with all perikarya appearing to contribute fibers to the plexus or the subciliary axon tract (Fig. 6A-C). Specific pairs of cells are distinct and therefore can be recognized from one larva to the next. Three major nerves emerge on each side of the plexus. The first forms a descending loop beneath the ciliated band before leading up into the antero-lateral arm, the second extends around the larval mouth, and the third appears to extend into the larval body on either side of the esophagus (Fig. 4D). The smaller 5HT-Li IR nerves in the larval arms may also feed into the apical ganglion, although the precise termination of these fibers was not determined. The overall shape of the serotonergic elements in the apical ganglion was characterized by cell bodies arranged anterior and posterior to the central plexus, but never very far ventral or dorsal to it (Fig. 6A-C). Therefore, it appears as a rather two-dimensional structure in confocal rotations.

The bilateral symmetry of the S1-Li IR components of the apical ganglion is less pronounced than that of the 5HT-Li IR components. Cell bodies are arranged along the length of the ganglion, rather than in clusters at either end (Fig. 6D-F). All views of the ganglion appear to show a variety of cell shapes. Some are flask-shaped, with tapering apical processes typical of sensory cells, whereas others are more rounded. Upon rotation, the rounded cells appear as

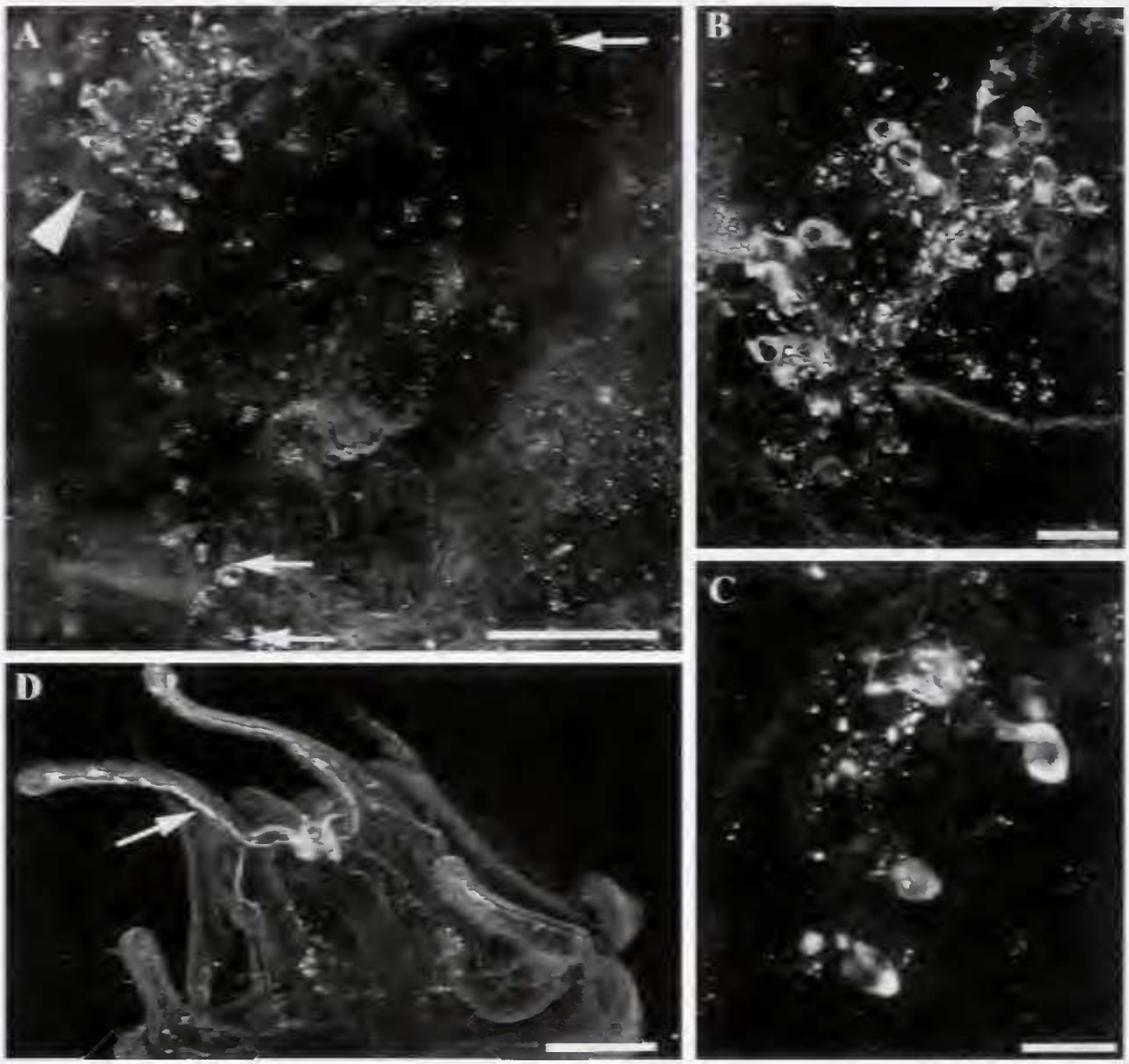

Figure 4. The serotonergic apical ganglion and circumoral complexes viewed by confocal laser scanning microscopy. (A) View of both the apical ganglion (arrowhead) and oral complexes and connecting fibers (arrows) in an 8-armed larva. (B and C) The apical ganglion and upper lip complexes are connected, but occupy different planes. The stack of confocal images used to construct (A) has been split to separate the cells of the apical ganglion (B) from those in the upper lip (C). (D) A 6-armed larva showing the limited ventral distribution of 5HT-Li IR. Major serotonergic nerve tracts can be seen exiting from the apical ganglion (arrow), passing into the anterolateral arms, around the mouth to the lower lip, and down into the larval body. Bars: A and D = 50 μm, B and C = 30 μm.

cross sections of flask-shaped cells, arranged at right angles to the others (Fig. 6D-F). Several major S1-Li IR axon tracts emerge from either side of the apical ganglion, projecting towards the larval arms and rudiment.

The larval pyloric neural complex

A new site of S1-Li IR was first detected in 10- to 11-day-old larvae (4- to 6-armed). A single fiber encircles the posterior end of the stomach at the pylorus (Fig. 7A) and is associated with a distinctive elongate cell body (Fig. 7B). The fiber is about 1 μm in diameter with 3 μm varicosities, and the bipolar cell body is 10-15 μm long. An optical section through the pylorus shows S1-Li IR on either side, although the fiber does not appear to be a ring. This circumpyloric nerve proves to be the origin of a nerve complex that increases in size throughout the rest of larval life.

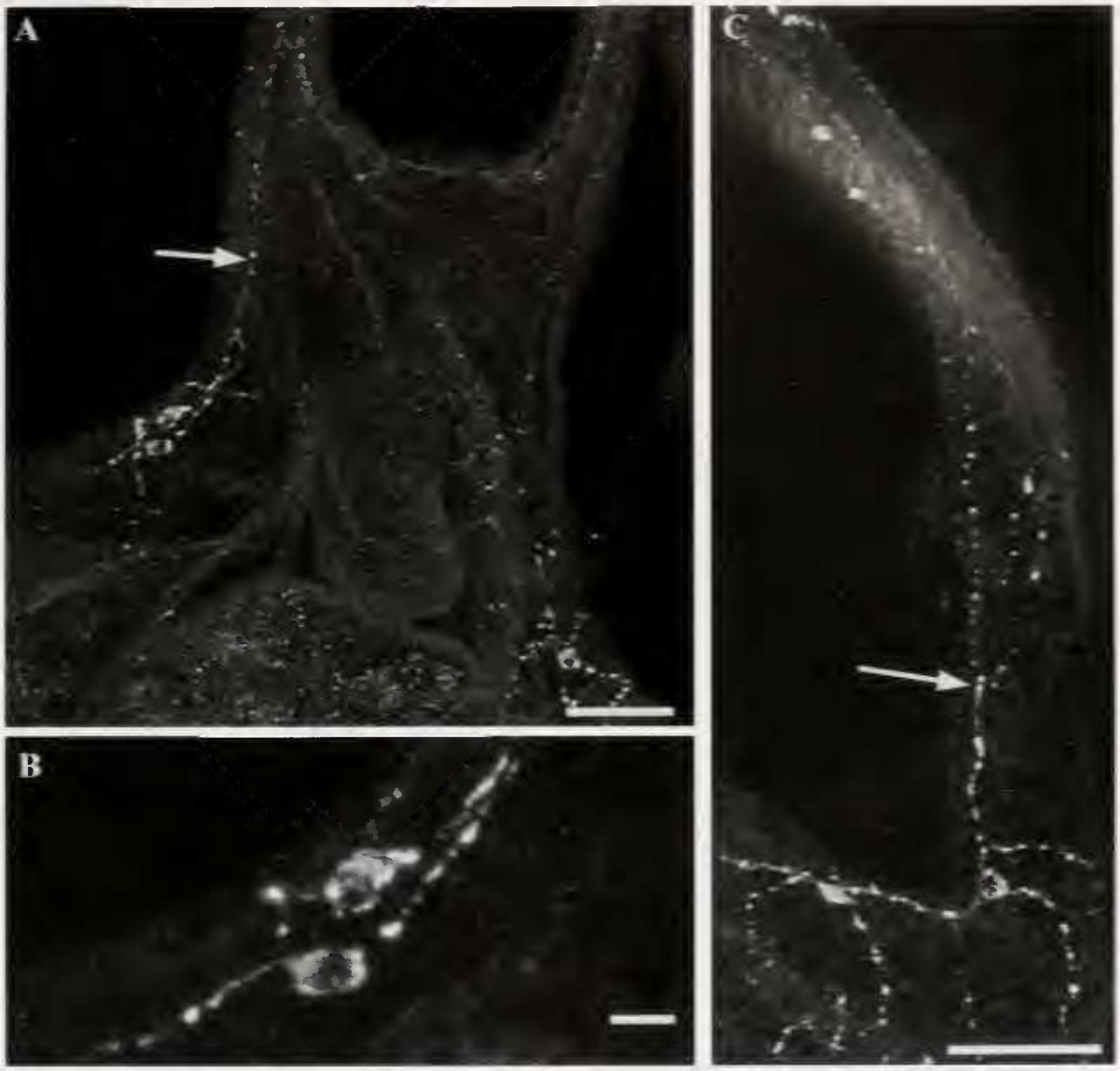

Figure 5. S2-Li IR associated with the ciliary band in a 6-armed larva viewed with confocal laser scanning microscopy. (A) S2-Li IR varicose axons underlying the ciliated band (arrow) and connecting to large immunoreactive neuronal cell bodies. (B) High power of the cell bodies shown in (A), lying between the anterolateral and posterodorsal arms. (C) S2-Li IR fibers extend into the larval body from neurons between the arms, as well as into the ciliary nerve (arrow) of the post-oral arms. Bars, A and C = 50 μm, B = 10 μm.

forming a loose web of fibers around much of the distal end of the stomach. Several days after the appearance of the first pyloric nerve, a second S1-Li IR neuron appears with a similar form to the first (Fig. 7C). More neurons are gradually added throughout development, and this complex continued to grow even in mature larvae where metamorphosis was delayed, apparently due to nutritional limitations. For example, in 50-day-old larvae, the pyloric neural complex consists of numerous cells and a plexus of fibers (Fig. 7D). No 5HT-Li IR was observed in this area.

The adult rudiment and metamorphosis

In early attempts to label the adult rudiment, the larval complexes were clearly immunoreactive, but nothing could be seen in the rudiment itself. Initial interpretations were that neurons in the rudiment either had not differentiated or did not express neuropeptide. However, semithin toluidine blue sections demonstrated the presence of developing radial nerve cords and the circumoral nerve ring. Therefore, the permeabilization procedure was modified in an attempt

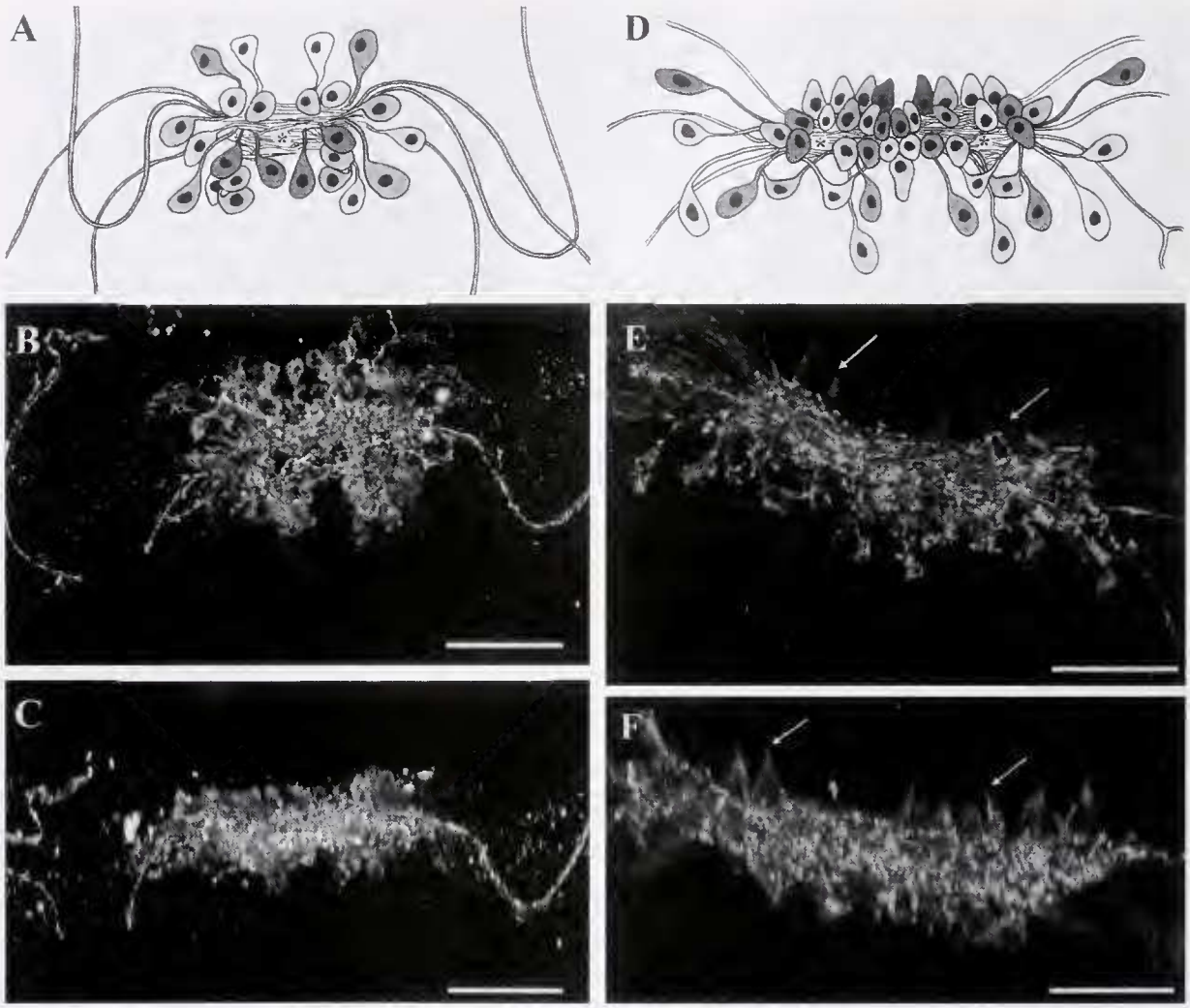

Figure 6. Representative drawings of 5HT and S1-Li IR neurons in the apical complex, and confocal images of the apical ganglion showing selected rotations from a full 360° series. (A) to (C) show the serotonergic cells of the apical ganglion. (A) Diagram of the apical ganglion viewed from the anterior/ventral surface of the larva, as deduced from the three-dimensional rotations. Not to scale. (B) 0° rotation. (C) 65° rotation. (D) to (F) show the SALMFamidergic cells of the apical ganglion. (D) Diagram of the SALMFamidergic cells of the apical ganglion as deduced from three-dimensional rotations. Not to scale. The ganglion is bilaterally symmetrical, apparently with pairs of cells (arrows). (E) 0° rotation. (F) 50° rotation; *indicates the nerve plexus. Bars = 25 μm.

to confirm unequivocally that the absence of immunoreactivity is due to the lack of antigen. This test still revealed positive labeling in several structures of the rudiment.

The first appearance of SALMFamide-like immunoreactivity in the rudiment of the larvae was in the ectodermally derived lining of the vestibular cavity (Fig. 8A). Some S1-Li IR appeared in the covering of the primary podia, which is also ectodermal in origin. The earliest S1-Li IR in the circumoral and radial nerve cords was rather diffuse. In later specimens competent to metamorphose (the primary podia were observed to move independently), the immunoreactivity was more concentrated, occurring in well-defined fiber tracts in the rays of the water vascular system, with the nerve ring in the tube feet clearly visible (Fig. 8B, C). No 5HT-Li IR was observed in the rudiment.

Discussion

The larval nervous system

This study describes the neural development of *Psammechinus miliaris,* from the 4-armed pluteus stage through to a metamorphically competent larvae with 8 arms and a developed adult rudiment. Most important, it extends the previously available descriptions of the echinoid larval nervous system to the late pluteus, and includes such previously unknown fea-

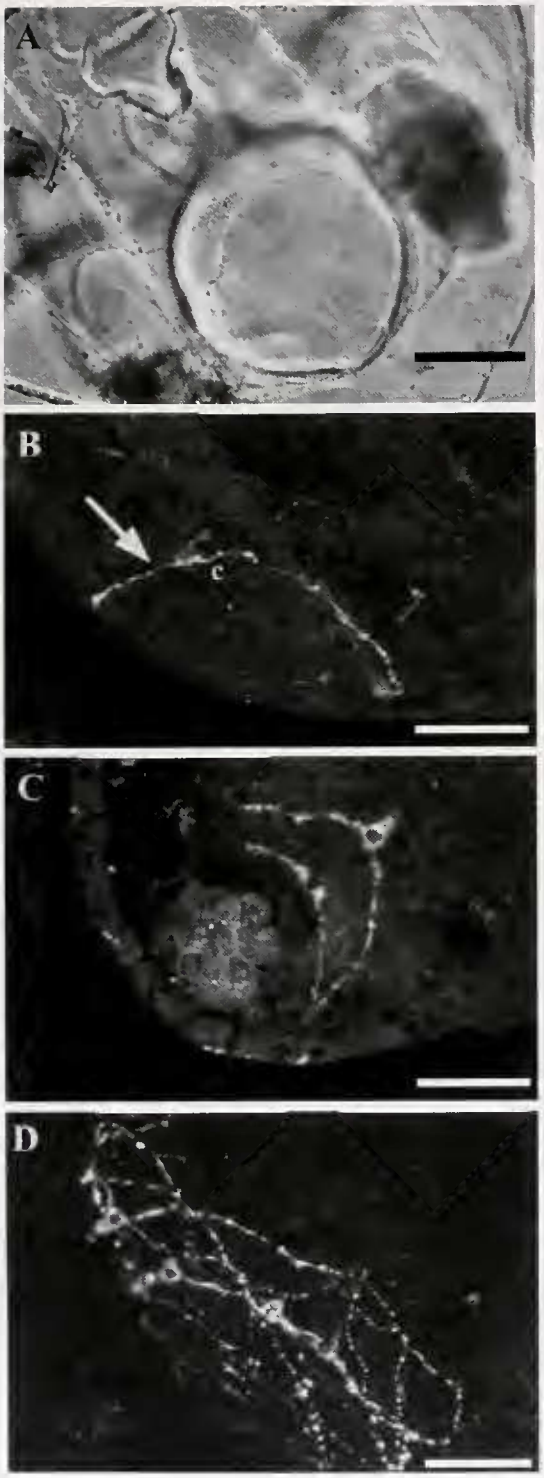

Figure 7. S1-Li IR in the pyloric neural complex. (A) 8-armed larva showing the pylorus. (B) Confocal image of the first immunoreactive cell (c) and fiber (arrow) in the complex in a 6-armed larva (10 days post-fertilization). (C) Two immunoreactive cells are present in the pyloric area by 16 days post-fertilization. (D) A plexus of cells develops in mature larvae, especially those which have delayed metamorphosis. Bars: A = 100 μm, B to D = 50 μm.

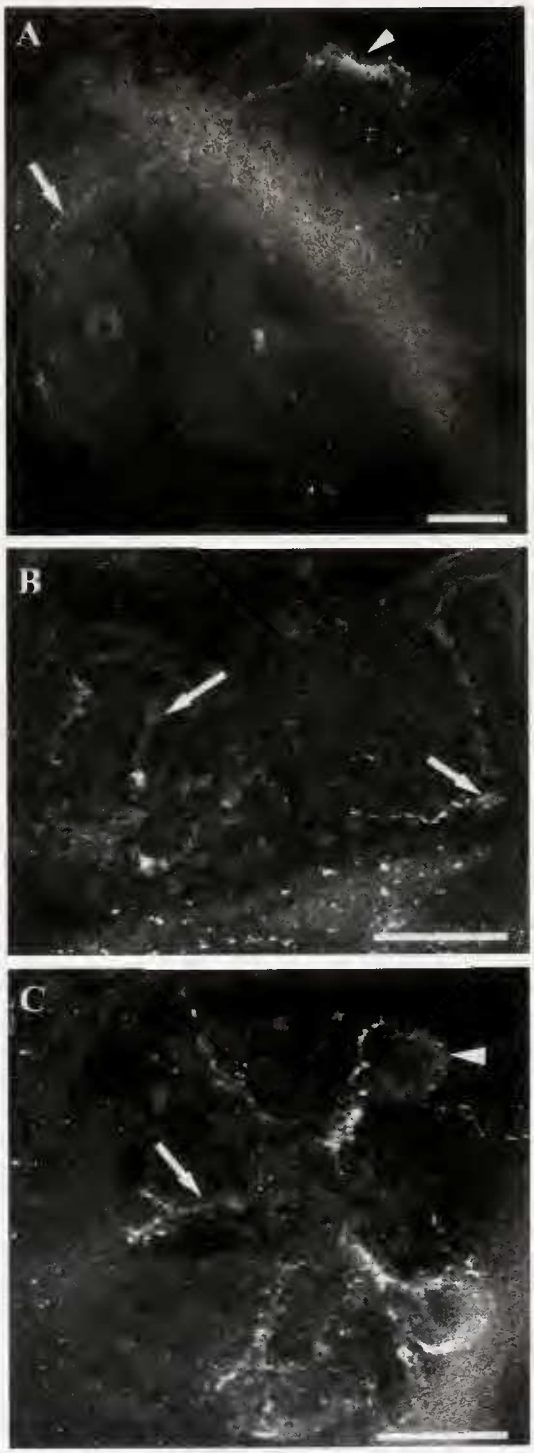

Figure 8. S1-Li IR in the adult rudiment. (A) Immunoreactivity can be seen in the apical ganglion of the larva (arrowhead) and in the vestibule wall of the rudiment (arrow) of a 27-day-old larva. (B) The earliest evidence of S1-Li IR in the developing radial nerves. Fibers in the neuro-epithelium underlying the epineural space lead into the primary podia (arrows). (C) S1-Li IR shows the radial nerve cords (arrow) terminate in the podial rings of the primary tube feet (arrowhead). Bars = 100 μm.

tures as the pyloric plexus and details of the nervous system within the adult rudiment. It also provides the fullest description to date of the apical ganglion in mature larvae. This ganglion is a complex structure containing numerous cell types, with connecting fibers to other organs within the larval body, including the adult rudiment.

The structure of certain elements of the echinoderm larval nervous system has been previously determined by ultrastructural, histological, and histochemical studies, revealing a network of cell bodies and fibers that are regionally concentrated to form ganglia (Burke, 1978; Nakajima, 1986; Bisgrove and Burke, 1987; Lacalli *et al.*, 1990; Moss *et al.*, 1994; Byrne *et al.*, 1999; Chee and Byrne, 1999). The ciliary bands are a major component of the larval nervous system and contain numerous nerve cells of several types. Other components include localized specializations such as in the lower lip, the apical (or dorsal) ganglion, and an underlying major nerve tract, the ciliary nerve. Immunocytochemical studies have been especially useful in providing details of the overall structure of this system; in the absence of a single unambiguous marker, previous authors have combined information from several immunological markers to construct a neural map. Our latest neural map of echinoid larvae is one further step towards a better understanding of the structure and function of the echinoderm nervous system.

The development of the nervous system of *P. miliaris* correlates with changes in larval form, function, and behavior. Additional neurons often appear in association with newly formed or enlarged larval organs. For example, the ciliated band continues to grow in length until the fourth and final pair of arms, the pre-oral arms, are formed. According to Nakajima (1986), this band also increases in width, the number of rows of ciliated cells increasing with age, especially in the epaulettes. The nerves underlying the ciliated band elongate as it grows, and additional immunoreactive cell bodies appear. The apical ganglion continues to grow as larvae approach competency, with the addition of immunoreactive neurons and an associated increase in complexity in the neuropile and ciliary nerves surrounding the area. We show that neural structures associated with the pylorus continue to develop, even in advanced larvae. This continual addition to the larval nervous system for the greater part of larval life suggests an increased behavioral repertoire, possibly specifically in association with the development of the adult rudiment and the timing of metamorphosis.

The development of 5HT and SALMFamide immunoreactivity

The early development of 5HT-Li IR in *P. miliaris* follows a pattern seen in other studies on echinoids (Bisgrove and Burke, 1987). Immunoreactive cells appear, and the number of stained cells increases as the larva begins to swim and feed. 5HT-Li IR is restricted to those components of the nervous system in the ventral half of the larva that are considered to be sensory—in particular, the apical ganglion and flask-shaped cells in the ciliary bands. The suggestion of a predominantly sensory function for 5HT in *P. miliaris* is in keeping with other studies on larval echinoderms (Bisgrove and Burke, 1987; Nakajima, 1988; Moss *et al.*, 1994), molluscs (Kempf *et al.*, 1997; Marois and Carew, 1997), and phoronids (Hay-Schmidt, 1990). The role of 5HT may be associated with the detection of chemical cues, possibly those required to induce feeding or settlement and metamorphosis. However, the absence of 5HT in the rudiment and juvenile may also be significant. The podia of the rudiment have been implicated in substrate choice by metamorphosing sea urchins (Cameron and Hinegarder, 1978), and no 5HT was detected in these structures in the current study. This suggests that if 5HT is involved in settlement behavior, it may be as part of a pathway that integrates both the larval and juvenile nervous systems.

The development of S1-Li IR follows a pattern similar to that of 5HT, and its temporal appearance can also be seen to coincide with increased locomotory activity and feeding. Its final distribution is wider than that observed for 5HT, with numerous cells and fibers throughout the larval body, as well as in the newly described pyloric plexus. The shape and location of cells in the pyloric plexus and subciliary complexes is highly suggestive of a motor function, whereas the tapering apical processes that bristle around the apical ganglion may represent sensory receptors. This mixed function has also been suggested by other studies in which S1 has been localized (Thorndyke *et al.*, 1992; Moss *et al.*, 1994), and it is possibly an early indicator of their ubiquitous distribution in adult echinoderms (Newman, 1995a, b). It is interesting that the preliminary studies with anti-S2 revealed staining only in the ciliary bands and a few neuronal cell bodies, but none in the ganglia or gut. This may suggest a purely motor function for S2 in coordinating ciliary activity and is again in keeping with the more limited distribution of this peptide in adult echinoderms (Newman, 1995a, b). As the work on S2 was only carried out on one developmental stage, we cannot draw definitive conclusions about its overall distribution. However, it does provide a clear comparison to the S1 distribution and reinforces the likelihood that these two related peptides have very distinct functions in both larvae and adults.

Co-localization of SALMFamide peptides and 5HT was not examined. The distributions revealed in this study clearly point towards discrete populations of cells expressing a single neurotransmitter. However, comparison of the two staining patterns also suggests that co-localization within specific cells of the ganglia is possible. Indeed, S1 has an extensive distribution in larval echinoderms and is probably of equal importance to larval function as 5HT. Equivalent patterns of neuropeptide localization in other

marine larvae have been reported for the molluscan neuropeptide FMRFamide, which is also found in central larval ganglia and peripheral nerves in larval phoronids (Hay-Schmidt, 1990). Otherwise the reporting of neuropeptide localization in the ganglia of marine invertebrate larvae is rare, and we are not yet able to draw any conclusions about their putative roles.

The apical ganglion

The apical ganglion is the major neural complex in the pluteus and probably has a central coordinating role. An apical neural structure, with varying degrees of complexity, has been implicated in the coordination of swimming, feeding, and settlement behavior in many marine invertebrate larvae, including molluscs (Chia and Koss, 1984; Marois and Carew, 1997; Kempf *et al.*, 1997), polychaetes (Lacalli, 1981), and echinoderms (Burke, 1983; Chia *et al.*, 1986; Nakajima *et al.*, 1993). This structure appears to be conserved in these larvae, and a consistent feature is the presence of 5HT-Li IR neurons, usually in a bilaterally symmetrical arrangement.

In the present study, CSLM has been used to trace the development of the apical ganglion and to analyze its structural complexity. At the 8-armed larvae stage it is by far the most complex neural structure in the larva, with an array of immunopositive cells and possibly other as yet undetected neurons. Although it appears highly developed, it presumably degenerates completely at metamorphosis, as it appears to coordinate only larval functions. In sea urchins, therefore, the entire larva seems to be under the control of a necessarily complex apical ganglion, which supports its functions in totality. In other words, the larval and juvenile nervous systems have been thought of as being almost totally distinct.

The apical ganglion may have a wider significance as a precursor of the chordate nervous system (Lacalli, 1994). Cell types within the larval nervous system have been compared with those in other phyla in an attempt to find homologous structures, implicating the larval nervous system, and especially the apical ganglion, as phylogenetically significant structures. The extensive ganglion revealed in 8-armed *P. miliaris* larvae may help shed further light on this possibility. Echinoderms, as deuterostome invertebrates, are useful for tracing such cells in chordates to their putative invertebrate ancestors. For example, Lacalli and West (1993) propose that the distinctive multipolar cells of echinoderm circumoral ciliary bands are homologous with similar neurons in the ciliary bands of the hemichordate tornaria, as well as with those in the dorsal nerve cord of ammocoete larvae of the lancelet *Branchiostoma*.

Development of immunoreactivity in the rudiment, and the fate of the larval nervous system

SALMFamide probes have revealed the presence of neuropeptides in the rudiment of the *P. miliaris* pluteus, indicating that adult neural development and differentiation is well under way during larval stages. This is the first immunocytochemical visualization of nerve cells in the developing rudiment and shows the clear separation of the larval and juvenile systems.

The discovery of the pyloric plexus in the older larvae is of particular interest since it has not been previously described. The structure continues to grow in mature larvae (and may continue to grow after metamorphosis), whereas other larval neural components have stopped growing, or may even be diminishing. These observations suggest that the pyloric plexus neurons may be incorporated into the adult basi-epithelial gut plexus. This is perhaps not surprising, since this area of the gut is incorporated into the rudiment along with associated nerves. Such a finding would indicate that some larval neural tissue is carried into the juvenile, with the major part of this system being resorbed at metamorphosis. However, the larval pyloric plexus and that of the juvenile may also be distinct structures that simply show expression of the same neuropeptide. Further studies focusing on this specific area may reveal more details of the metamorphic process and the relationship between larval and adult forms.

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Literature Cited

Bisgrove, B. W., and R. D. Burke. 1987. Development of the nervous system of the pluteus larva of *Strongylocentrotus droebachiensis*. *Cell Tissue Res.* **248:** 335–343.

Burke, R. D. 1978. The structure of the larval nervous system of the pluteus larva of *Strongylocentrotus purpuratus*. *Cell Tissue Res.* **191:** 233–247.

Burke, R. D. 1983. Neural control of metamorphosis in *Dendraster excentricus*. *Biol. Bull.* **164:** 176–188.

Byrne, M., F. Chee, P. Cisternas, and M. C. Thorndyke. 1999. Localisation of the neuropeptide S1 in the larval and adult nervous system of the sea star *Patiriella regularis*. Pp. 187–191 in *Echinoderm Research 1998*, M. D. Candia Carnevali and F. Bonasoro, eds. Balkema, Rotterdam.

Cameron, R. A., and R. T. Hinegarder. 1978. Early events in sea urchin metamorphosis, description and analysis. *J. Morphol.* **157:** 21–31.

Candia Carnevali, M. D., F. Bonasoro, U. Welsch, and M. C. Thorndyke. 1998. Arm regeneration and growth factors in crinoids.

Pp. 145–150 in *Echinoderms: San Francisco*, R. Mooi and M. Telford, eds. Balkema, Rotterdam.

Chee, F., and M. Byrne. 1999. Development of the larval serotonergic nervous system in the sea star *Patiriella regularis* as revealed by confocal imaging. *Biol. Bull.* **197:** 123–131.

Chia, F.-S., and R. Koss. 1984. Fine structure of the cephalic sensory organ in the larva of the nudibranch *Rostanga pulchra*. *Zoomorphology* **104:** 131–139.

Chia, F.-S., R. D. Burke, R. Koss, P. V. Mladenov, and S. S. Rumrill. 1986. Fine structure of the doliolaria larva of feather star, *Florometra serratissima*, with special emphasis on the nervous system. *J. Morphol.* **189:** 99–120.

Díaz-Miranda, L., D. A. Price, M. J. Greenberg, T. D. Lee, K. E. Doble, and J. E. García-Arrarás. 1992. Characterization of two novel neuropeptides from the sea cucumber, *Holothuria glaberrina*. *Biol. Bull.* **182:** 241–247.

Elphick, M. R., R. H. Emson, and M. C. Thorndyke. 1991a. Isolation of the neuropeptide SALMFamide-1 from starfish using a new antiserum. *Peptides* **12:** 455–459.

Elphick, M. R., D. A. Price, T. D. Lee, and M. C. Thorndyke. 1991b. The SALMFamides: a new family of neuropeptides isolated from an echinoderm. *Proc. R. Soc. Lond. B* **243:** 121–127.

Ghyoot, M., J. L. Cobb, and M. C. Thorndyke. 1994. Localisation of neuropeptides in the nervous system of the brittlestar *Ophiura ophiura*. *Philos. Trans. R. Soc. Lond. B* **346:** 433–444.

Hay-Schmidt, A. 1990. Distribution of catecholamine-containing, 5HT-like and FMRFamide-like IR neurons and processes in the nervous system of the actinotroch larvae of *Phoronis muelleri*. *Cell Tissue Res.* **259:** 105–118.

Iwata, K. S., and H. Fukase. 1964. Comparison of discharge of the gametes by three artificial means in sea urchins. *Biol. J. Okayama Univ.* **10:** 57–64.

Kelly, M. S., A. J. Hunter, C. L. Scholfield, and J. D. McKenzie. 2000. Morphology and survivorship of larval *Psammechinus miliaris* (Gmelin) (Echinodermata: Echinoidea) in response to varying food quantity and quality. *Aquaculture* **183:** 223–240.

Kempf, S. C., L. R. Page, and A. Pires. 1997. Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch molluscs with emphasis on the structure and possible function of the apical sensory organ. *J. Comp. Neurol.* **386:** 507–528.

Kroll, R. P., and E. E. Voronezhskaya. 1996. Early elements in gastropod neurogenesis. *Dev. Biol.* **173:** 344–347.

Lacalli, T. C. 1981. Structure and development of the apical organ in trochophores of *Spirobranchus polycerus*, *Phyllodoce maculata* and *Phyllodoce mucosa* (Polychaeta). *Proc. R. Soc. Lond. B* **212:** 381–402.

Lacalli, T. C. 1994. Apical organs, epithelial domains and the origins of the chordate central nervous system. *Am. Zool.* **34:** 533–541.

Lacalli, T. C., and J. E. West. 1993. A distinctive nerve cell type common to diverse deuterostome larvae: comparative data from echinoderms, hemichordates and amphioxus. *Acta Zool.* **74:** 1–8.

Lacalli, T. C., T. H. Gilmour, and J. E. West. 1990. Ciliary band innervation in the bipinnarian larva of *Pisaster ochraceus*. *Philos. Trans. R. Soc. Lond. B* **330:** 371–390.

MacBride, E. W. 1913. The development of *Echinocardium cordatum* I. External features of development. *Q. J. Microsc. Sci.* **59.**

MacBride, E. W. 1914. *A Textbook of Embryology*. Macmillan Press, London.

MacBride, E. W. 1918. The development of *Echinocardium cordatum* II. *Q. J. Microsc. Sci.* **63.**

Mackie, G. O., A. N. Spencer, and R. Strathmann. 1969. Electrical activity associated with ciliary reversal in an echinoderm larva. *Nature* **223:** 1384–1385.

Marois, R., and T. J. Carew. 1997. Fine structure of the apical ganglion and its serotonergic cells in the larva of *Aplysia californica*. *Biol. Bull.* **192:** 388–398.

Mortensen, T. H. 1921. Studies on the development and larval forms of echinoderms. G.E.C. Gad, Copenhagen.

Moss, C., R. D. Burke, and M. C. Thorndyke. 1994. Immunocytochemical localisation of the neuropeptides S1 and serotonin in larvae of the starfish *Pisaster ochraceus* and *Asterias rubens*. *J. Mar. Biol. Assoc. UK* **74:** 61–71.

Nakajima, Y. 1986. Development of the nervous system of sea urchin embryos: Formation of the ciliary bands and the appearance of two types of ectoneural cells in the pluteus. *Dev. Growth Differ.* **28:** 531–542.

Nakajima, Y. 1988. Serotonergic nerve cells of starfish larvae. Pp. 235–239 in *Echinoderm Biology*, R. D. Burke, P. V. Mladenov, P. Lambert, and R. L. Parsley, eds. Balkema, Rotterdam.

Nakajima, Y., R. D. Burke, and Y. Nnda. 1993. The structure and development of the apical ganglion in the sea urchin pluteus larvae of *Strongylocentrotus droebachiensis* and *Mespilia globulus*. *Dev. Growth Differ.* **35:** 531–538.

Newman, S. J., M. R. Elphick, and M. C. Thorndyke. 1995a. Tissue distribution of the SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens* using novel monoclonal and polyclonal antibodies. I. Nervous and locomotory systems. *Proc. R. Soc. Lond. B* **261:** 139–145.

Newman, S. J., M. R. Elphick, and M. C. Thorndyke. 1995b. Tissue distribution of the SALMFamide neuropeptides S1 and S2 in the starfish *Asterias rubens* using novel monoclonal and polyclonal antibodies. II. Digestive system. *Proc. R. Soc. Lond. B* **261:** 187–192.

Polak, J. M., and S. Van Noorden. 1986. *Immunocytochemistry. Modern Methods and Applications*. John Wright and Sons, Bristol, United Kingdom.

Potton, D. 1997. Neuroendocrine control of feeding in the European starfish, *Asterias rubens*. Ph.D. thesis, University of London, United Kingdom.

Strathmann, M. F. 1987. Reproduction and development of marine invertebrates from the northern Pacific coast: data and methods for the study of eggs, embryos and larvae. University of Washington Press, Seattle.

Thorndyke, M. C., B. D. Crawford, and R. D. Burke. 1992. Localisation of a SALMFamide neuropeptide in the larval nervous system of the sand dollar, *Dendraster excentricus*. *Acta Zool.* **73:** 207–212.